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Arand, Michael ; Marowsky, Anne

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Michael Arand, Anne Marowsky
Institute of Pharmacology and Toxicology, University of Zurich (CH);

The endocannabinoid 2-arachidonyl glycerol (2-AG) is substantially hydrolysed by at least two enzymes, fatty acid amide hydrolase (FAAH) and monoarachidonyl glycerol lipase (MAGL), which thereby terminate its biological activity. In a recent report it has been claimed that microsomal epoxide hydrolase (mEH), hitherto known as a xenobiotic detoxifying enzyme, also rapidly catalyses the breakdown of 2-AG. However, the catalytic site architecture of mEH argues against an esterase activity. We therefore analyzed the capacity of recombinant purified human, mouse and rat mEH to hydrolyze 2-AG. In contrast to the previous finding, we find only marginal 2-AG esterase activity (≤ 50 nmol/mg protein/min) associated with the purified enzymes that was resistant to inhibition by the potent mechanism-based mEH inhibitor 1,1,1-trichloropropene 2,3-oxide (TCPO). Likewise, 2-AG hydrolysis in mouse liver microsomes was resistant to TCPO inhibition while being efficiently blocked by methyl arachidonyl fluorophosphonate (MAFP). MAFP, on the other hand, failed to inhibit epoxide hydrolase activity of both, purified mEH and mouse liver microsomes. We therefore conclude that mEH lacks any appreciable 2-AG hydrolase activity.

Correspondence
arand@pharma.uzh.ch

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Objective

We therefore set out to re-assess the potential activity of mEH as a 2-AG hydrolase by directly testing this activity with purified mEH.

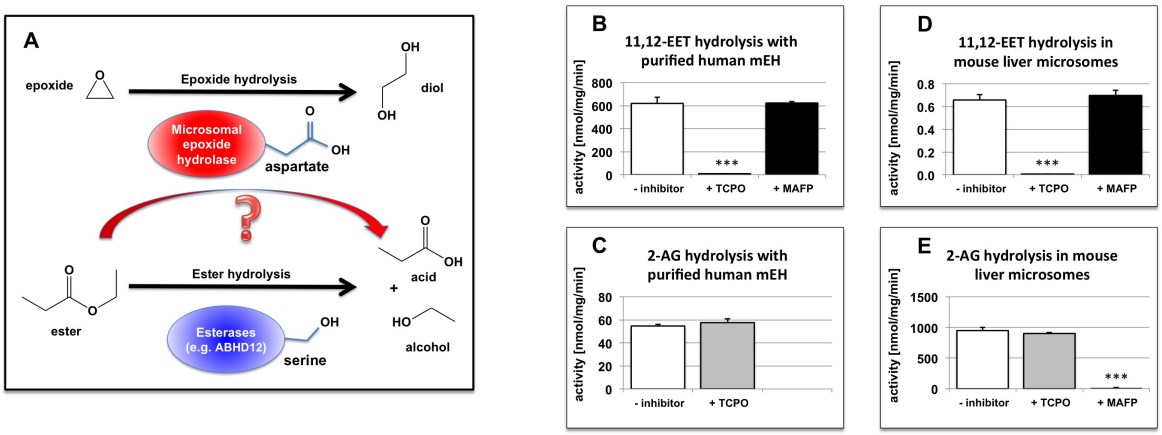
Introduction

In a recent publication, Nithipatikom and colleagues **(Nithipatikom 2014[1])** claimed that the xenobiotic-metabolising enzyme microsomal epoxide hydrolase (mEH) **(Oesch 1973[2])** efficiently hydrolyses the endocannabinoid 2-arachidonyl glycerol (2-AG) **(Mechoulam 1995[3])**. Endocannabinoid signaling is important for a variety of physiological processes such as pain sensation **(Hohmann 2005[4])**, cognition and emotion **(Crowe 2014[5])** and appetite regulation **(Di Marzo_2001[6])**. Thus the capability of mEH to control 2-AG levels would have substantial implications, also because mEH is almost ubiquitously expressed throughout the human body **(Coller 2001[7])**.

The study by **(Nithipatikom 2014[1])** was inspired by the fact that mEH belongs to the structural superfamily of α/β hydrolase fold enzymes **(Zou 2000[8])** and therefore shares structural similarity to a wide range of esterases and lipases **(Lenfant 2013[9])**. Two of these, ABHD6 and ABHD12, have recently been shown to posses 2-AG hydrolase activity **(Blankman 2007[10])**.

In their experiments, the authors used microsomes of cell lines (over)expressing mEH. Microsomes are membrane preparations obtained by differential centrifugation of cell or tissue homogenates and contain essentially all the membrane-associated proteins that are not confined to large organelles such as nuclei and mitochondria. Therefore, the contribution of mEH to the total protein content of these microsomes probably does not exceed 1%. Unfortunately, the authors do not give quantitative measures for this, but the fact that they use 30 µg microsomal protein for their Western blot analyses is in agreement with the above estimate. In their turnover experiments, the authors observed 2-AG hydrolysis in microsomes that, after subtraction of the significant background reaction observed in non-transfected material, correlated with the degree of mEH expression and was responsive to siRNA mediated mEH down regulation and inhibition by small molecules, the most potent of these being methyl arachidonyl fluorophosphonate (MAFP). From their results, they conclude that mEH is a novel 2-AG hydrolase.
Some facts cast doubt on the interpretation of the study: 1) Although mEH is indeed a member of the α/β hydrolase fold enzyme superfamily, it belongs to a sub-class that differs

in the most important component of its catalytic machinery from esterases and lipases:
While the active site catalytic nucleophile of lipases/esterases is a serine that forms a covalent intermediate with the acid component of its ester substrates **(Sussman 1991[11])**, epoxide hydrolases carry an aspartic acid at this position, forming an ester intermediate with the alcoholic component of its substrates **(Lacourciere 1993[12])** (Fig. 1A; Fig. S1 for a more detailed explanation).2) The control membrane preparations used in the study posses already a high background activity for 2-AG hydrolysis that is not attributable to mEH, FAAH or MAGL, and the increase in activity, where it can be taken from the authors presentation, is only a 2 - 3-fold gain, 3) the strongest inhibitor identified in the study, MAFP, is known as a powerful inhibitor of PLA2-type lipases **(Lio 1996[13])**, yet no potency to inhibit mEH has so far been demonstrated, and finally 4) neither 2-AG hydrolytic activity nor sensitivity to MAFP inhibition of purified mEH have been tested by the author, which would be a definitive proof for their claim.



(A) The separate types of α/β hydrolase fold enzymes use different catalytic residues to hydrolyse different types of substrates: while epoxide hydrolases cleave the ether bond in epoxides with an aspartic acid side chain, esterases hydrolyse esters using a serine side chain (for further details see Fig. S1). Epoxide hydrolase activity of purified human mEH (B) and of mouse liver microsomes (D) is sensitive to TCPO inhibition and resistant to MAFP treatment. In contrast, 2-AG hydrolase activity of purified human mEH (C) and of mouse liver microsomes (E) is resistant to TCPO treatment, while this activity in liver microsomes is strongly inhibited by MAFP. The column heights give the mean of 3 separate measurements. Error bars indicate the standard deviation. *** $p < 0.001$
NB: MAFP sensitivity of 2-AG hydrolase activity associated with the human mEH purified from bacteria was not tested because of the lack of relevance: the bacterial enzyme obviously contributing this activity (see our collective results) may or may not be inhibited by the compound. Either result would not have any impact on our conclusions.

Results & Discussion

Recombinant expression and His-tag-based metal chelate chromatography purification yielded purified mouse, rat and human mEH protein that displayed turnover rates with the prototypic substrate 11,12-epoxyeicosatrienoic acid (11,12-EET) of 0.1, 0.3 and 0.65 μmol per mg protein per minute, respectively. On SDS polyacrylamide gel electrophoresis and subsequent Coomassie staining the recombinant proteins displayed the expected dominant signal at 52 kDa (Fig. S2A), with some minor impurities amounting to 14, 18 and 27% for the rat, mouse and human enzyme, respectively, as determined by densitometric quantification.

2-AG turnover with purified rat, mouse and human mEH revealed relatively poor, apparently TCPO-resistant enzymatic activity (Fig. S2B-G) that roughly correlated with the degree of impurity of the enzyme preparation. The most active preparation was that of human mEH with an estimated turnover rate of 50 nmol arachidonic acid formed per milligram protein per minute. Because the activity reported by **(Nithipatikom 2014[1])** for human mEH was several orders of magnitudes higher (see last paragraph of this section for a comparison), these results already demonstrate that mEH does not possess substantial 2-AG hydrolase activity. However, to clarify whether the measurable 2-AG hydrolase activity in our recombinant purified enzymes might be attributed to any impurities of the preparation rather than to mEH itself, we refined the analysis of enzymatic turnover in the presence and absence of 1,1,1-trichloropropene 2,3-oxide (TCPO), a potent mEH inhibitor. This compound acts as a suicide substrate, characterised by an extremely slow hydrolysis rate of the covalent intermediate formed in the first step. Therefore, any activity of the enzyme requiring the catalytic nucleophile is efficiently blocked by a sufficient TCPO concentration. In presence of 1 mM TCPO, 99% of the human mEH enzymatic activity were blocked using 4 uM 11,12-EET as substrate (Fig. 1B; the Km for human mEH with 11,12-EET is reported to be 0.4 μM **(Decker 2012[14])**). By contrast, 2-AG hydrolysis by human mEH remained essentially unchanged with 100 μM 2-AG (Fig. 1C; the Km with 2-AG is 40 μM according to **(Nithipatikom 2014[1])**). This shows that 2-AG hydrolytic activity is not associated with the mEH protein itself, but is most probably due to minor impurities of bacterial esterases in our enzyme preparation. In addition, we tested MAFP for its capability to block mEH hydrolase activity. With an IC50 of 7.9 nM this compound was identified as the most potent inhibitor by **(Nithipatikom 2014[1])** to block what they proposed to be mEH-mediated 2-AG hydrolysis. However, 11,12-EET hydrolysis by purified human mEH remained unaffected in the presence of 10 μM MAFP (Fig. 1B).

To test whether the native membrane environment in the endoplasmic reticulum provides essential components for any mEH-mediated 2-AG hydrolytic activity which might explain the lack of the respective enzymatic activity with the purified enzyme, we next analysed 2-AG and 11,12-EET hydrolysis in microsomes prepared from the livers of sEH -/- mice, lacking the mEH sister enzyme soluble epoxide hydrolase (sEH), the presence of which might otherwise interfere with the efficacy of TCPO to inhibit 11,12-EET hydrolysis. In the absence of any inhibitor, the microsomes showed the expected hydrolytic activities with 11,12-EET (0.66 nmol/mg/min; Fig 1D) and 2-AG (950 nmol/mg/min; Fig 1E). Again, EET hydrolysis was highly sensitive to TCPO inhibition and resistant to MAFP treatment (Fig. 1D), while the opposite effect was observed with 2-AG hydrolysis (Fig 1E).

The 2-AG hydrolase activity reported by **(Nithipatikom 2014[1])** is very high. From Fig. 4 of their manuscript, an enzymatic activity of around 300 nmol 2-AG being hydrolyzed per minute and milligram microsomal protein can be deduced. Given the above estimate that at best 1% of the microsomal enzyme can be attributed to mEH, this would imply that mEH has a specific 2-AG hydrolase activity of ≥ 30 μmol per milligram per minute. We do not find activity with purified mEH from either rat, mouse or human in excess of 50 nmol per milligram per minute. On top, the inhibitor sensitivity of both, the 2-AG hydrolysis by purified mEH enzyme as well as of mouse liver microsomes, is incompatible with its origin from mEH, but suggests that even this minor activity in our preparations originates from bacterial enzymes that have not completely separated from the mammalian proteins during purification.

Conclusions

Our results clearly demonstrate that mEH lacks any appreciable 2-AG hydrolase activity. With respect to the observations made by **(Nithipatikom 2014[1]),** we suggest as an alternative explanation that mEH might activate a so far uncharacterized MAFP-sensitive 2-AG hydrolase.

Conjectures

The next obvious step is to identify the 2-AG hydrolase that is apparently activated by mEH, as well as the mechanism by which this activation takes place. According to the report by Nithipatikom et al., this hydrolase can neither be FAAH nor MAGL. In our view, the potential candidates are ABHD6, ABHD12, CES1 and CES2, due to their reported significant capacity to hydrolyze 2-AG **(Blankman 2007[10]) (Xie 2010[15])**.

Additional Information

Methods and Supplementary Material

Please see https://medicalmatters.io/articles/201605000008.

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Ethics Statement

Not applicable

Citations

[1] K. Nithipatikom et al. "A novel activity of microsomal epoxide hydrolase: metabolism of the endocannabinoid 2-arachidonoylglycerol". In: *The Journal of Lipid Research* 55.10 (June 2014), pp. 2093–2102. DOI: 10.1194/jlr.m051284. URL: http://dx.doi.org/10.1194/jlr.m051284.

[2] Franz Oesch. "Mammalian Epoxide Hydrases: Inducible Enzymes Catalysing the Inactivation of Carcinogenic and Cytotoxic Metabolites Derived from Aromatic and Olefinic Compounds". In: *Xenobiotica* 3.5 (Jan. 1973), pp. 305–340. DOI: 10.3109/00498257309151525. URL: http://dx.doi.org/10.3109/00498257309151525.

[3] Raphael Mechoulam et al. "Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors". In: *Biochemical Pharmacology* 50.1 (June 1995), pp. 83–90. DOI: 10.1016/0006-2952(95)00109-d. URL: http://dx.doi.org/10.1016/0006-2952(95)00109-d.

[4] Andrea G. Hohmann et al. "An endocannabinoid mechanism for stress-induced analgesia". In: *Nature* 435.7045 (June 2005), pp. 1108–1112. DOI: 10.1038/nature03658. URL: http://dx.doi.org/10.1038/nature03658.

[5] Molly S. Crowe et al. "The endocannabinoid system modulates stress, emotionality, and inflammation". In: *Brain, Behavior, and Immunity* 42 (Nov. 2014), pp. 1–5. DOI: 10.1016/j.bbi.2014.06.007. URL: http://dx.doi.org/10.1016/j.bbi.2014.06.007.

[6] Vincenzo Di Marzo et al. In: *Nature* 410.6830 (Apr. 2001), pp. 822–825. DOI: 10.1038/35071088. URL: http://dx.doi.org/10.1038/35071088.

[7] Janet K. Coller et al. In: *The Histochemical Journal* 33.6 (2001), pp. 329–336. DOI: 10.1023/a:1012414806166. URL: http://dx.doi.org/10.1023/a:1012414806166.

[8] Jinyu Zou et al. "Structure of Aspergillus niger epoxide hydrolase at 1.8 Å resolution: implications for the structure and function of the mammalian microsomal class of epoxide hydrolases". In: *Structure* 8.2 (Feb. 2000), pp. 111–122. DOI: 10.1016/s0969-2126(00)00087-3. URL: http://dx.doi.org/10.1016/s0969-2126(00)00087-3.

[9] Nicolas Lenfant et al. "Proteins with an alpha/beta hydrolase fold: Relationships between subfamilies in an ever-growing superfamily". In: *Chemico-Biological Interactions* 203.1 (Mar. 2013), pp. 266–268. DOI: 10.1016/j.cbi.2012.09.003. URL: http://dx.doi.org/10.1016/j.cbi.2012.09.003.

[10] Jacqueline L. Blankman, Gabriel M. Simon, and Benjamin F. Cravatt. "A Comprehensive Profile of Brain Enzymes that Hydrolyze the Endocannabinoid 2-Arachidonoylglycerol". In: *Chemistry & Biology* 14.12 (Dec. 2007), pp. 1347–1356. DOI: 10.1016/j.chembiol.2007.11.006. URL: http://dx.doi.org/10.1016/j.chembiol.2007.11.006.

[11] J. Sussman et al. "Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein". In: *Science* 253.5022 (Aug. 1991), pp. 872–879. DOI: 10.1126/science.1678899. URL: http://dx.doi.org/10.1126/science.1678899.

[12] Gerard M. Lacourciere and Richard N. Armstrong. "The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate". In: *J. Am. Chem. Soc.* 115.22 (Nov. 1993), pp. 10466–10467. DOI: 10.1021/ja00075a115. URL: http://dx.doi.org/10.1021/ja00075a115.

[13] Yi-Ching Lio et al. "Irreversible inhibition of Ca2+-independent phospholipase A2 by methyl arachidonyl fluorophosphonate". In: *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* 1302.1 (July 1996), pp. 55–60. DOI: 10.1016/0005-2760(96)00002-1. URL: http://dx.doi.org/10.1016/0005-2760(96)00002-1.

[14] M. Decker et al. "EH3 (ABHD9): the first member of a new epoxide hydrolase family with high activity for fatty acid epoxides". In: *The Journal of Lipid Research* 53.10 (July 2012), pp. 2038–2045. DOI: 10.1194/jlr.m024448. URL: http://dx.doi.org/10.1194/jlr.m024448.

[15] Shuqi Xie et al. "Inactivation of Lipid Glyceryl Ester Metabolism in Human THP1 Monocytes/Macrophages by Activated Organophosphorus Insecticides: Role of Carboxylesterases 1 and 2". In: *Chem. Res. Toxicol.* 23.12 (Dec. 2010), pp. 1890–1904. DOI: 10.1021/tx1002194. URL: http://dx.doi.org/10.1021/tx1002194.

[16] Huey-Fen Tzeng, L. Timothy Laughlin, and Richard N. Armstrong. "Semifunctional Site-Specific Mutants Affecting the Hydrolytic Half-Reaction of Microsomal Epoxide Hydrolase †". In: *Biochemistry* 37.9 (Mar. 1998), pp. 2905–2911. DOI: 10.1021/bi9727388. URL: http://dx.doi.org/10.1021/bi9727388.

[17] A. Marowsky et al. "Distribution of soluble and microsomal epoxide hydrolase in the mouse brain and its contribution to cerebral epoxyeicosatrienoic acid metabolism". In: *Neuroscience* 163.2 (Oct. 2009), pp. 646–661. DOI: 10.1016/j.neuroscience.2009.06.033. URL: http://dx.doi.org/10.1016/j.neuroscience.2009.06.033.

[18] C. J. Sinal et al. "Targeted Disruption of Soluble Epoxide Hydrolase Reveals a Role in Blood Pressure Regulation". In: *Journal of Biological Chemistry* 275.51 (Sept. 2000), pp. 40504–40510. DOI: 10.1074/jbc.m008106200. URL: http://dx.doi.org/10.1074/jbc.m008106200.

[19] Apace, DI, and Franklin. "N-Benzylimidazole, a high magnitude inducer of rat hepatic cytochrome P-450 exhibiting both polycyclic aromatic hydrocarbon- and phenobarbital-type induction of phase I and phase II drug-metabolizing enzymes". In: *Drug Metab Dispos* 16 (1988), pp. 259–264.

Figure legends

Fig. S1 Structure and enzymatic mechanism of human mEH in comparison to the enzymatic mechanism of serine hydrolases, example ABHD12

A. Stereoview of a 3D model of human epoxide hydrolase showing the active site residues. The model is based on the structure of the related juvenile hormone epoxide hydrolase from *Bombyx mori* (Zhou et al. Proteins (2014) 82, 3224-3229). The respective PDB file is also provided as Supplementary Material (hmEH model.pdb) for further inspection.
B. The two step mechanism of enzymatic epoxide hydrolysis by mEH. In a first step of the catalysis, the catalytic nucleophile Asp226 attacks an electron-deficient carbon of the oxirane ring to form an ester intermediate. This intermediate is subsequently hydrolyzed by means of the water-activating His431-Glu404 pair, the so-called charge relay system. The two tyrosines Tyr290 and Tyr 374 are important hydrogen bond donors that activate the substrate molecule and keep it in favourable position for the attack by Asp226. Note that the acid component of the ester intermediate is provided by the enzyme.
C. The two step mechanism of enzymatic ester hydrolysis typical for serine hydrolases. The first step of enzymatic hydrolysis is a transesterification of the acidic component of the substrate molecule from its alcoholic part to the catalytic serine of the enzyme, Ser246 in ABHD12 (Navia-Paldanius et al. J. Lipid Res. (2012) 53, 2413-2424). Subsequent hydrolysis takes place analogous the the above mechanism with a similar charge relay system, His372-Asp333 in ABHD12. This time, the acid component of the ester intermediate originates from the substrate. The possibility to swap substrates between these two types of related yet substantially different mechanisms is not obvious.

Fig. S2 2-AG hydrolysis by purified mammalian mEHs in the absence and presence of the mEH-specific inhibitor TCPO

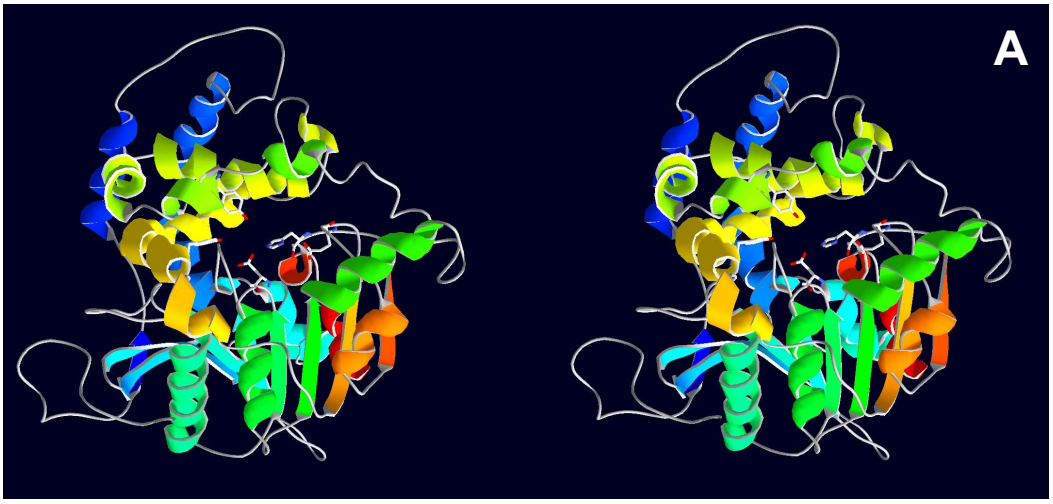
A. Visualization of purified rat (lanes 1 + 2), mouse (lanes 2 + 3) and human (lanes 5 + 6) mEH after His-tag purification. 4 µg of protein were loaded on each lane and the SDS-polyacryl-amide gel was stained with Coomassie brilliant blue after electrophoretic separation of proteins. Marker proteins (lane M) were used to monitor the apparent molecular weight of the sample proteins. Of the two separate purification batches of each protein, the one labelled by an arrow was used for the subsequent turnover experiments.
B. – G. Ion chromatograms of 2-AG turnover with rat (B, C), mouse (D, E) and human (F, G) mEH in the absence (B, D, F) and presence (C, E, G) of TCPO. The red arrow indicates the small peaks obtained for arachidonic acid liberated during enzymatic hydrolysis. Procedure 2 was used for chromatographic separation. Under these conditions, the substrate 2-AG is not detected.

Fig. S3 Refined analysis of 2-AG and 11,12-EET turnover by purified human mEH

A. – D. Ion chromatograms of 2-AG hydrolysis by purified human mEH (lane 5 in Fig. S2A). A and B show the signal for arachidonic acid (1 µM in assay buffer) and 2-AG (100 µM in assay buffer), respectively. C and D depicts the signal after turnover in the absence (C) and presence (D) of the mEH inhibitor TCPO. C2 and D2 are magnifications to better show the arachidonic acid signal intensity that is almost invisible at normal scale (C1 and D1). Chromatographic separation was performed using procedure 3. The red arrows indicate the position of arachidfonic acid.
E. – G. Ion chromatograms of 11,12-EET hydrolysis by the same enzyme preparation. E shows the substrate under starting conditions (4 µM) while F and G present the signal after substrate turnover in the absence (F) and presence (G) of TCPO. The strong signal reduction for the product 11,12-DHET in the presence of TCPO is obvious. Note, that also the scale has changed to higher sensitivity, due to the fact that the less sensitively detected substrate is now limiting the scale (note that, with the exception of 3C2 and 3D2, all chromatograms are presented as screen shots without adjustmet of the scale, for the sake of authenticity; only external rulers have been added to facilitate the reading of the scales). Chromatographic separation was performed using procedure 1. The red arrow indicates the position of 11,12-DHET.

Fig. S4 2-AG and 11,12-EET turnover by mouse liver microsomes

A. – C. Ion chromatograms of 2-AG hydrolysis by mouse liver microsomes without inhibitor (A), as well as in the presence of TCPO (B) or MAFP (C). The red arrow indicates the position of the product arachidonic acid. Chromatographic separation was performed using procedure 2.
D. – F. Ion chromatograms of 11,12-EET hydrolysis by mouse liver microsomes without inhibitor (D), as well as in the presence of TCPO (E) or MAFP (F). The red arrow indicates the position of the product 11,12-DHET. Chromatographic separation was performed using procedure 2.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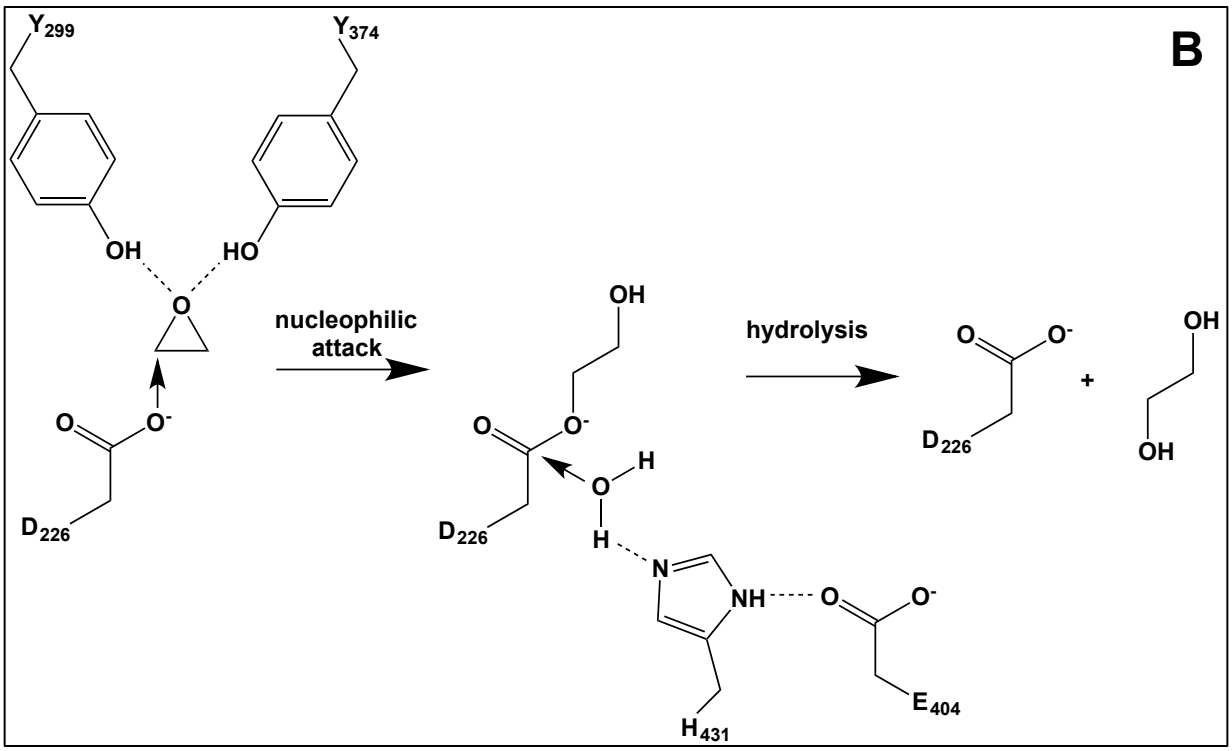
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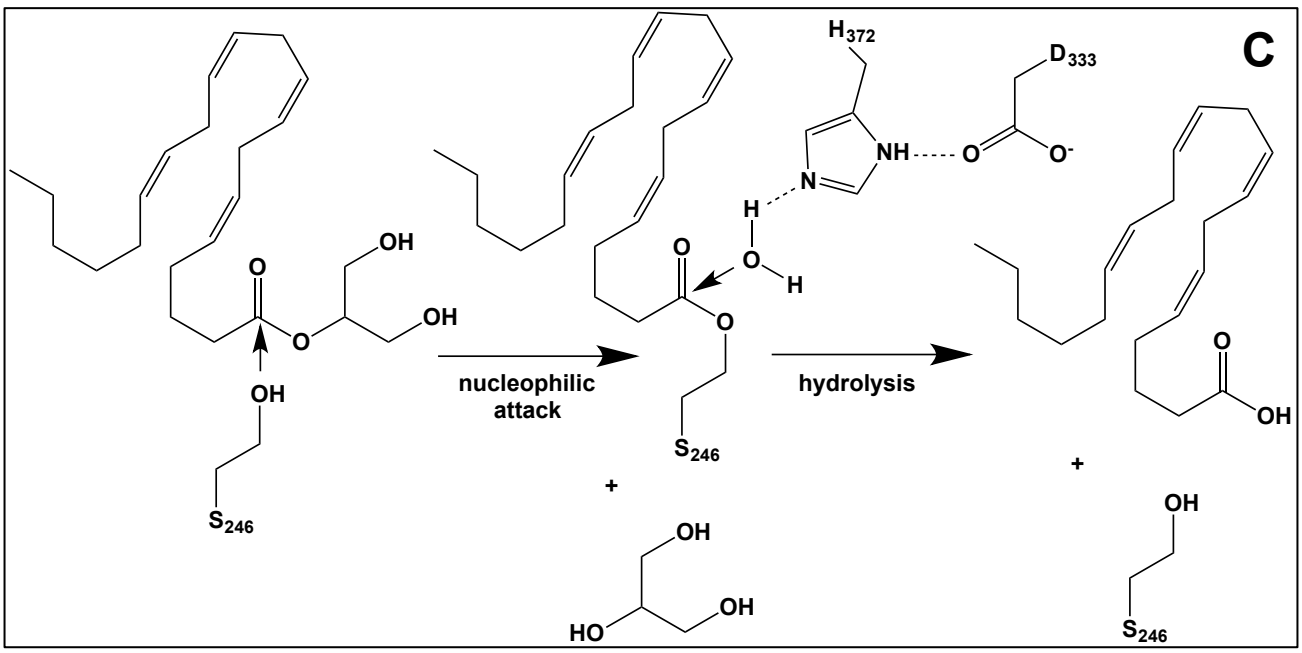
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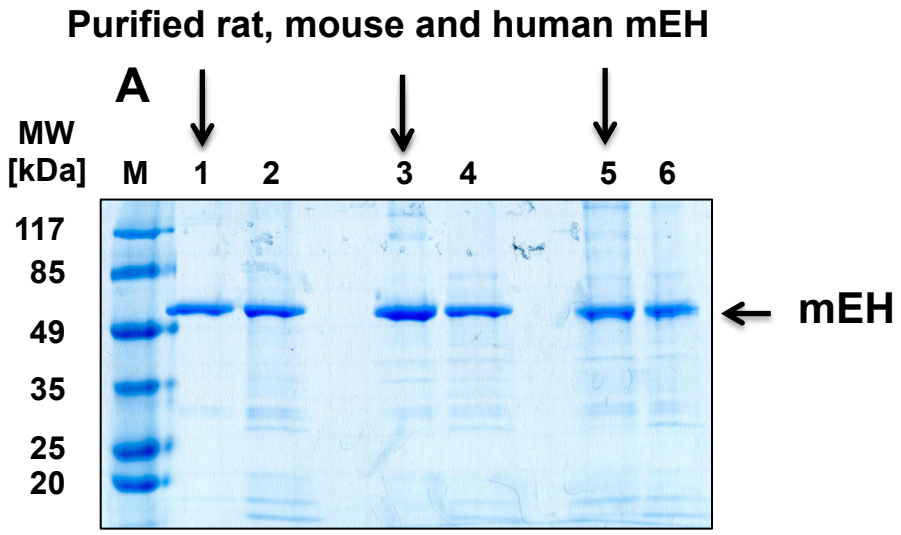
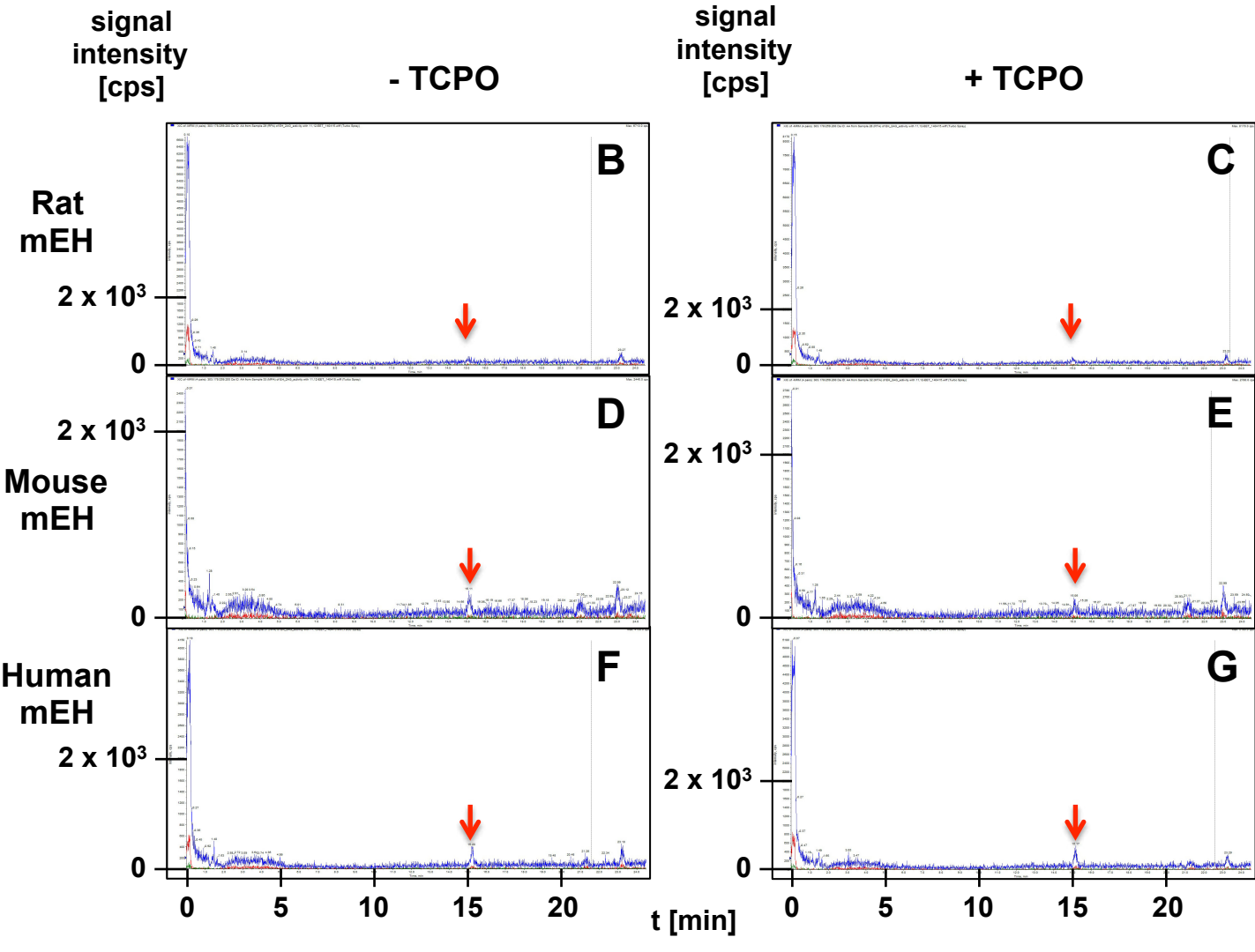
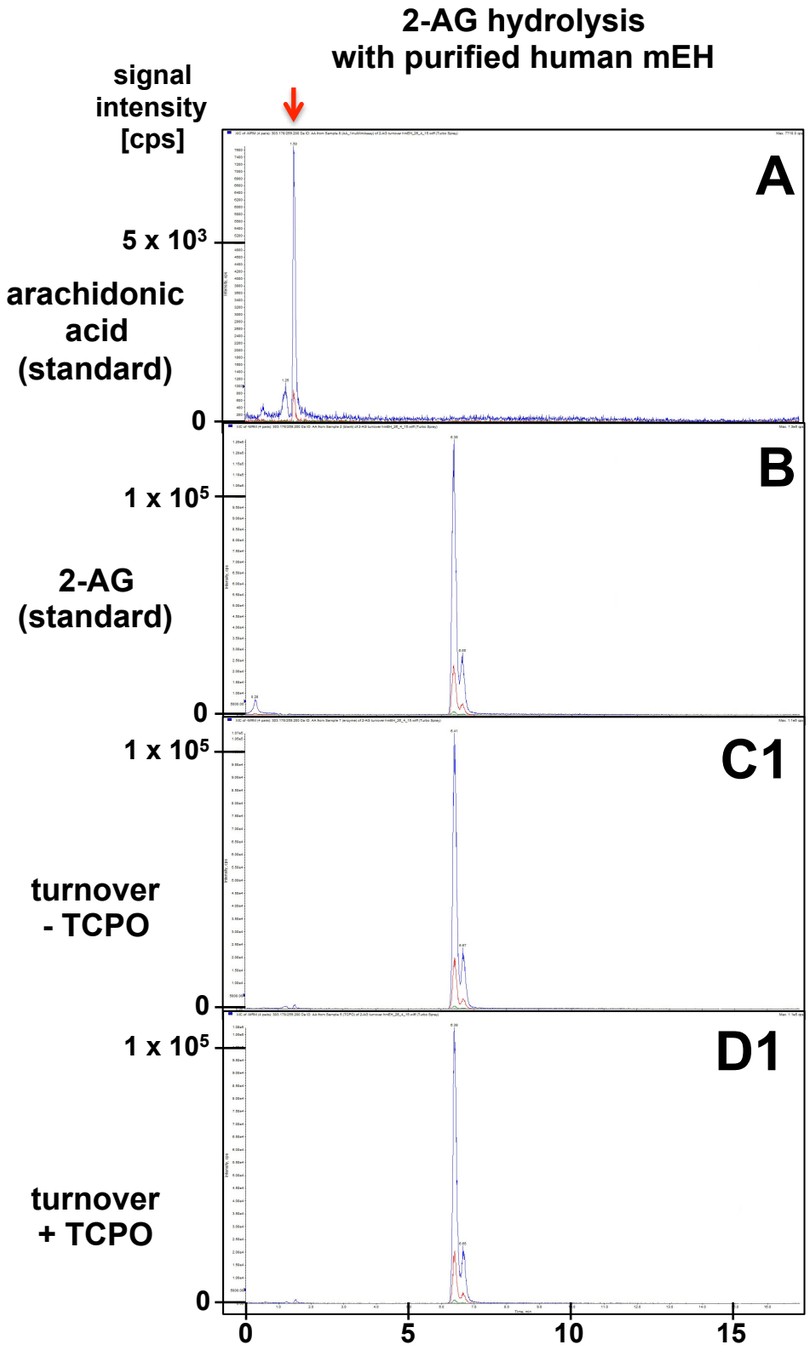
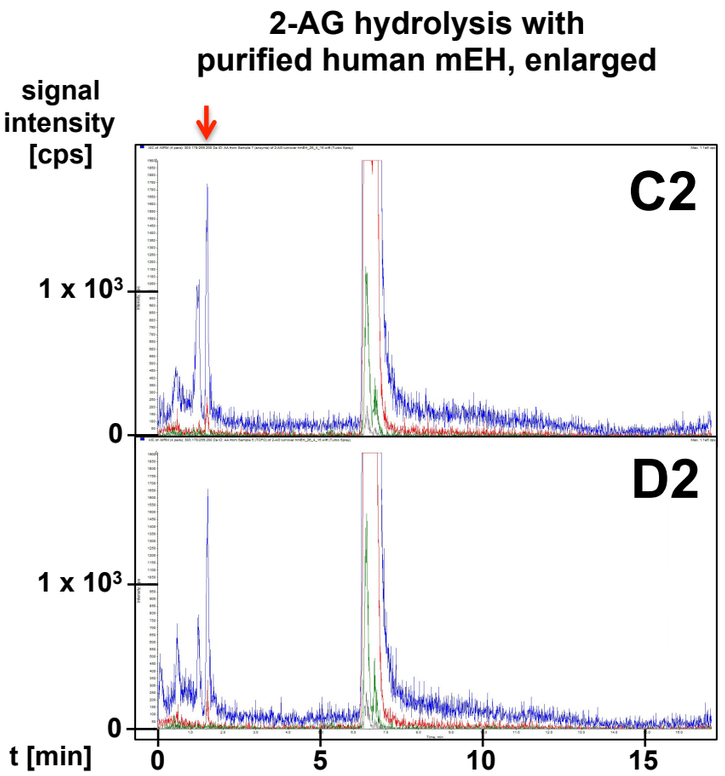
2-AG hydrolysis by purified mammalian mEHs in the absence and presence of the mEH-specific inhibitor TCPO

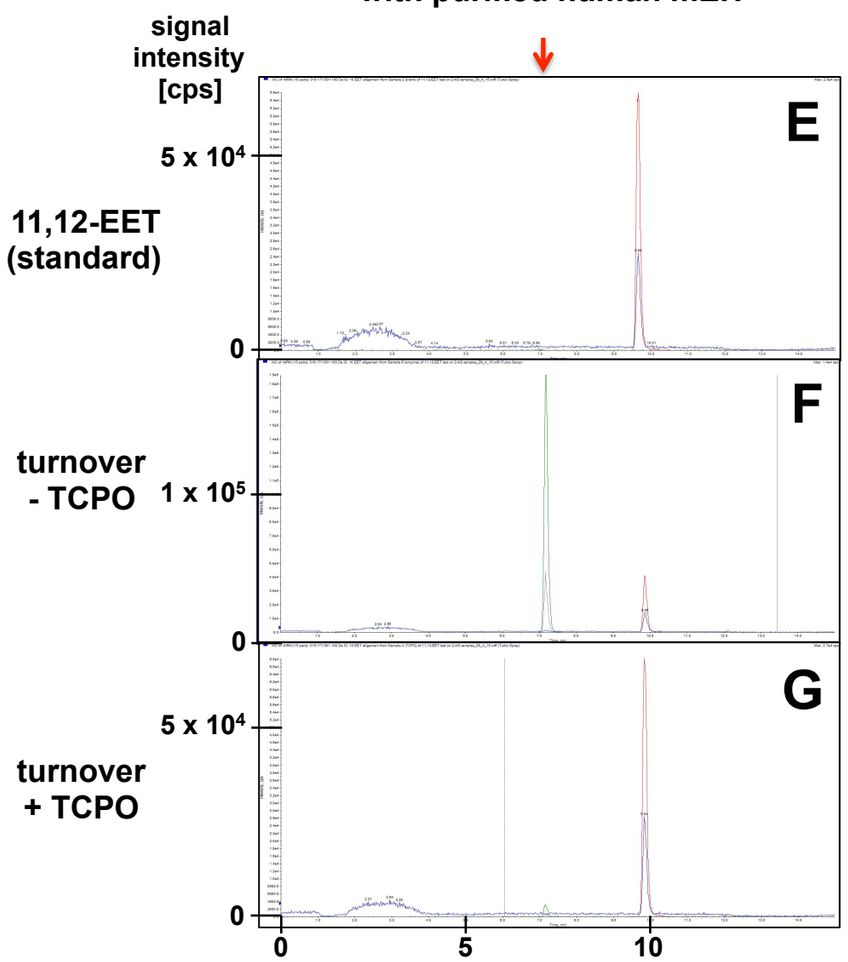




Refined analysis of 2-AG and 11,12-EET turnover by purified human mEH







2-AG and 11,12-EET turnover by mouse liver microsomes